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PHD2 exerts anti-cancer and anti-inflammatory effects in colon cancer xenografts mice via attenuating NF-κB activity

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Abstract

Recent studies suggested that prolyl hydroxylase 2 (PHD2) functions as an important regulator in vascular inflammation and *Streptococcus pneumonia* infection. However, whether PHD2 contributed to tumor progression prompted by intratumoral inflammation remains elusive. In this study, the effects of PHD2 in colon cancer were evaluated, and the underlying molecular mechanisms were investigated. The results showed that overexpressing PHD2 exerted proliferative and migratory inhibition in colon cancer cells. The expression of cell cycle and epithelial-mesenchymal transition (EMT)-associated proteins were changed: CyclinD1, CDK4, N-cadherin, and

Vimentin were down-regulated, while E-cadherin was up-regulated in PHD2-overexpressing colon cancer cells. Moreover, in colon cancer xenograft mice, PHD2 overexpression suppressed tumor growth accompanied by decreased Ki67 expression. Importantly, we further demonstrated that overexpressing PHD2 attenuated inflammation in colon cancer xenograft mice through weakening accumulation of myeloid-derived suppressor cells (MDSCs) and M2-like tumor-associated macrophages (TAMs), as well as secretions of pro-inflammatory cytokines including G-CSF, TNF-α, IL-6, IL-8, IL-1β, and IL-4. Mechanistically, PHD2 overexpression obviously suppressed NF-κB activity through decreasing phosphorylated IκB-α while increasing cytoplasmic NF-κB p65 levels in colon cancer. Our findings support the anti-cancer and anti-inflammatory roles of PHD2 and offer a preclinical proof of tumor progression regulated by cancer cells and inflammation.

Keywords: PHD2; Colon cancer; Inflammation; NF-κB

Introduction

Colon cancer is the third most common type of cancer worldwide (Bray et al. , 2018). The rapid recurrence and metastasis of colon cancer, despite optimal treatments, remain the main reasons for mortality (Wu, 2018). Emerging evidence indicates that tumor progression not only depends on the cancer cells themselves but also on an inflammatory tumor microenvironment (Quail and Joyce, 2013) and its regulation (Banerjee et al. , 2019).

Systemic and local chronic inflammation enhances the risk of developing colon cancer, and an inflammatory tumor microenvironment enhances the growth and metastatic potential of the cancer cells (Yao et al. , 2019). Sustained intestinal inflammation is associated with NF-κB activation, which in turn inhibits apoptosis and promotes cell growth during early tumorigenesis (Viennois et al. , 2013)(Karin, 2009). Activation of the NF-κB pathway also plays roles in the recruitment of inflammatory immune cells and immature myeloid-derived cells in the enterocyte

microenvironment, leading to a vicious circle that sustains the inflammatory cell microenvironment (Musolino et al. , 2017, Perfilyeva et al. , 2019, Yao, Dong, 2019). Among the immune cells recruited, tumor-associated macrophages (TAMs) participate in angiogenesis, matrix remodeling, invasion and metastasis, immune tolerance toward the cancer cells, and chemoresistance in solid tumors (Tariq et al. , 2017). Therefore, the inflammatory factors induce an immune tolerance state and participate in cancer progression (Karin, 2009, Viennois, Chen, 2013). Alleviating the inflammation in the tumor microenvironment might be a key to improve the response to treatment and improve the prognosis (Caetano et al. , 2016).

Prolyl hydroxylases domain proteins (PHDs) are dioxygenases closely associated with cellular response to oxygen availability by hydroxylating the conserved prolyl residues of the hypoxia-inducible factor α (HIF-1α) (Jokilehto and Jaakkola, 2010). The PHD family consists of three members, PHD1, PHD2, and PHD3, and each isoform displays its own tissue- and cell line-specific functions (Minervini et al. , 2015). Of the three PHDs, PHD2 contributes to the greatest part of the PHD activity and has a low expression in colon cancer, hepatocellular carcinoma, and melanoma (Di Conza et al. , 2017a, Tao et al. , 2016).

Recent studies indicate that PHD2 is a potential target for inflammatory diseases. Sadiku *et al.* showed that the myeloid-specific loss of PHD2 resulted in an exaggerated inflammatory response to *Streptococcus pneumonia* (Sadiku et al. , 2017). Fan *et al.* demonstrated that PHD2 depletion in endothelial cells protects mice from LPS-induced pulmonary injury and related inflammatory diseases (Fan et al. , 2019). Shang *et al.* reported recently that PHD2 knockdown suppressed inflammatory responses of periodontitis controlled by human gingival fibroblasts (Shang et al. , 2018). Therefore, PHD2 appears to play a pivotal role in anti-inflammation, and a study reported low levels of PHD2 in colon cancer (Rawluszko et al. , 2013), but the contribution of PHD2 to the inflammatory tumor microenvironment and tumor progression is unclear.

In this study, we aimed to investigate the anti-cancer and anti-inflammatory effects of PHD2 in colon cancer as well as exploring underlying molecular mechanisms.

Materials and Methods

Cell culture

The colon cancer cell lines CT26 (mesenchymal origin) and MC38 (epithelial origin) were obtained from the American Type Culture Collection (ATCC) and Kerafast, respectively. They are cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FBS, Biolnd, Israel) and 5% antibiotics (100 U/mL of penicillin, 100 mg/mL of streptomycin; Sigma, St Louis, MO, USA), and maintained in a humidified 5% CO_2 atmosphere incubator at 37°C. In this study, the passage number was <20, and the cell lines were stable.

Construction of PHD2-overexpressing colon cancer cells

Overexpressing sequences were designed (No. 13GS0330, Invitrogen Inc., Carlsbad, CA, USA), cloned into the pcDNA3.1 (+)-EGFP vector (Invitrogen Inc., Carlsbad, CA, USA), and transfected into the colon cancer cells by Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Colon cancer cells overexpressing PHD2 were selected by Blasticidin S HCl (Invitrogen Inc., Carlsbad, CA, USA) at 4 μg/mL. The cloned cells were named PHD2 CT26 or PHD2 MC38, respectively. Similarly, the pcDNA3.1 (+)-EGFP plasmid served as a negative control (Invitrogen Inc., Carlsbad, CA, USA) and was named Mock CT26 or Mock MC38.

MTT assay

The cells were seeded at 2×10^3 per well on 96-well plates, cultured for 1-5 days, and subjected to the MTT assay. Briefly, the MTT (5 mg/mL) reagent (Sigma, St Louis, MO, USA) was added to each well, and the plates were incubated for 4 h in a humidified 5% CO_2 atmosphere incubator at 37°C until formazan crystals were formed. Then, the crystals were dissolved in DMSO, and the absorbance at 570 nm

was measured with a microplate reader (Bio-Rad, Hercules, CA, USA), using wells without cells as blanks. Cell viabilities were presented as the % survival rate, using the following formula: % survival rate = A_{570} of treated cells/A_{570} of control cells×100%.

Wound healing assay

The cells were grown to confluent monolayers on 6-well plates, and a pipette tip was used to create linear wounds. Wound images were captured and counted from five randomly selected fields under a photo camera-equipped light microscope (Leica Microsystems, Wetzlar, Germany).

Transwell assay

Cell invasion was detected using Transwell chambers (8-μm pore size; Millipore corp., Billerica, MA, USA) with a Matrigel (BD Biosciences, Franklin Lake, NJ, USA) matrix. Cells (1×10^5 in 200 μL) in serum-free DMEM were seeded on the top chamber, and 600 μL of DMEM with 10% FBS were added to the bottom chamber. After 48 h, the cells on the top membrane were mechanically removed using a cotton swab, and the cells on the bottom surface of the membrane were fixed in 4% paraformaldehyde and stained with a 4 g/L crystal violet solution.

MDSC migration was detected using Transwell chambers (5-μm pore size; Corning Inc., Corning, NY, USA). Cells (1×10^4 in 200 μL) in serum-free DMEM were seeded on the top chamber and 600 μL of serum-free DMEM containing 100 ng/mL rmG-CSF (PeproTech, Rocky Hill, NJ, USA) or/and 10 nM TNF-α (R&D Systems, Minneapolis, MN, USA) were added to the bottom chamber. After 12 h, the MDSCs on the top membrane were mechanically removed using a cotton swab, and MDSCs on the bottom surface of the membrane were fixed in 4% paraformaldehyde and stained with 4 g/L crystal violet solution. All the samples were captured and counted from five randomly selected fields under a photo camera-equipped inverted microscope (Leica Microsystems, Wetzlar, Germany).

Establishment of colon cancer xenografts mice and ethics statement

All experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University (SCU, Chengdu, China) (animal approval #2018121A). All

animal feeding and experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee at SCU.

CT26 cells or PHD2 CT26 cells (5×10^5 in 100 μL of PBS) were inoculated in the right mid-dorsal flank of male BALB/c mice (8-week-old, Hua Fu Kang Biotechnology Co. Ltd., Beijing, China, n=4/group). Five weeks after inoculation, the mice were sacrificed, and the tumor tissues were removed and weighed. Tumor volume (mm^3) was calculated and estimated as length × $width^2/2$.

Flow cytometry assay

The PHD2 CT26 and PHD2 MC38 cells were collected, and the proportions of GFP-positive colon cancer cells were determined using flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lake, NJ, USA).

For cell cycle detection, the cells were harvested and fixed in cold 70% ethanol at 4°C overnight. Subsequently, the cells were incubated with 50 ng/mL PI staining solution and 0.1 mg/mL RNase A in the dark for 30 min at room temperature. The DNA content of these cells was analyzed using flow cytometry.

For MDSCs and TAMs, single-cell suspensions derived from xenografts were collected and stained with antibodies targeting murine CD11b (APC-labeled), Gr1 (PE-labeled), F4/80 (APC-labeled), CD206 (PE-labeled), or an appropriate isotype control antibody (eBioscience, San Diego, CA, USA), and then analyzed by flow cytometry.

Hochest33342 staining

Cells were seeded in 96-well plates at 2×10^3 per well overnight and incubated with 5 μg/mL Hochest33342 (Beyotime Institute of Biotechnology, Haimen, China) for 15 min. After that, cells were washed twice with PBS and observed under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Enzyme-linked immunosorbent assay

Commercial mouse ELISA kits (Neobioscience, Shenzhen, China) were used to evaluate the levels of pro-inflammatory cytokines in xenografts or cell culture supernatant. For xenografts, 100 mg of protein lysate of xenografts were collected. For cell culture supernatants, 100 μl of supernatant were collected from cultured cells.

ELISAs for IL-8, TNF-α, IL-6, IL-4, IL-1β, and G-CSF were performed according to the manufacturer's instructions.

Immunofluorescence

The xenografts were fixed in 4% paraformaldehyde and embedded in OCT compound (Tissue Tek; Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) at 4°C. Frozen section (4-μm thick) were plated on matrigel-coated glass coverslips, permeabilized with 0.5%Triton X-100 for 10 min at room temperature, washed with PBS, and blocked in 5% BSA for 30 min before incubation with primary rabbit antibody (Proteintech Group inc., Chicago, IL, USA) against Ki67 (1:100) overnight at 4°C, followed by fluorescence-tagged secondary antibody against rabbit IgG (1:500). Hochest33342 was used for nuclear staining. Images were obtained using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Western blot

Cells or tumor samples were collected and treated with the Nuclear and Cytoplasmic Protein Extraction Kit or RIPA lysis buffer supplemented with protease inhibitor cocktail and protein phosphatase inhibitor (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein were separated by 8%-2% SDS-PAGE and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore corp., Billerica, MA, USA) by electroblotting. The membranes were probed with specific antibodies including PHD2, E-cadherin, N-cadherin, Vimentin (1:500; Abcam, Cambridge, MA, USA), GAPDH, β-actin, and Histone H3 (1:500; Beijing Biosynthesis Biotechnology Co., LTD, Beijing, China), pho-IκBα, IκBα, pho-NF-κB p65, NF-κB p65, TNF-α, Bax, Bcl-2, CyclinD1, CDK4, MMP2, and MMP9 (1:500; Cell Signaling, Danvers, MA, USA). The blots were developed with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibody (1:10,000; ZSGB-BIO; Beijing, China). The bands were visualized using the Western Chemiluminescent HRP Substrate (ECL; Millipore; Billerica, MA, USA) detection system. The band intensities were quantified by densitometry using the Image J software (version 1.50i; National Institutes of Health; Bethesda, MD, USA).

Statistical analysis

Data were presented as means ± standard deviations (SD) from at least three separate experiments and analyzed using GraphPad Version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Significant differences among groups were analyzed by the Student's *t*-test or one- or two-way ANOVA, as appropriate. Differences were considered significant if the P-value was <0.05.

Results

Construction of PHD2-overexpressing PHD2 CT26 and PHD2 MC38 cells

PHD2 CT26 and PHD2 MC38 cells were constructed through plasmid transfection. As shown in Fig 1A-D, the transfected efficiency of pcDNA3.1 (+)-EGFP and pcDNA3.1 (+)-EGFP-overexpressing PHD2 in CT26 and MC38 cells were significantly high, which was more than 90% by flow cytometry detection. Furthermore, western blot analysis demonstrated that the expression levels of PHD2 in PHD2 CT26 and PHD2 MC38 cells were increased compared to Mock CT26 and Mock MC38 cells or CT26 and MC38 cells (Fig 1E-H).

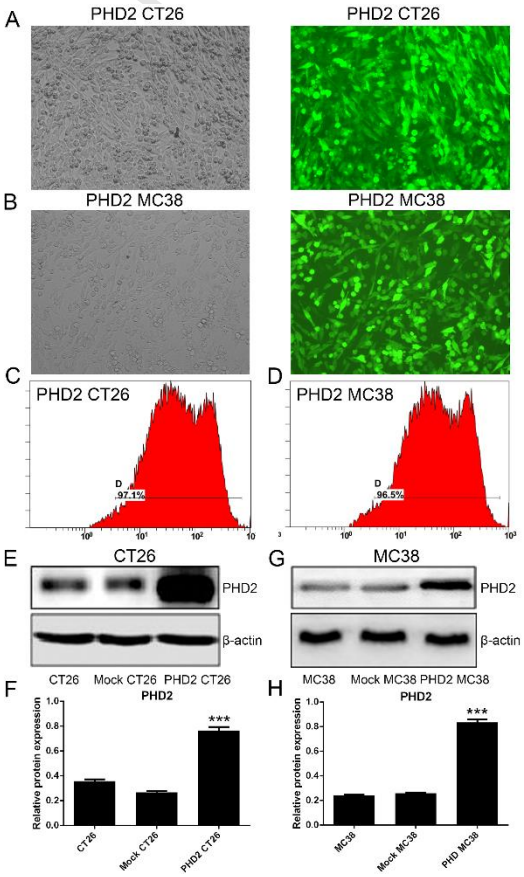


Figure 1. Transfected efficiency of overexpressing PHD2 in CT26 and MC38 cells. A. GFP-positive PHD2 CT26 cells under the fluorescence microscope. B. GFP-positive PHD2 MC38 cells under the fluorescence microscope. C. Proportion of GFP-positive PHD2 CT26 cells by flow cytometry. D. Proportion of GFP-positive PHD2 MC38 cells by flow cytometry. E. Western blot for the expression levels of PHD2 CT26 cells. F. Quantification of E. G. Western blot for the expression levels of PHD2 MC38 cells. H. Quantification of G. Original magnification: ×200. PHD2 CT26 and PHD2 MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. *** P<0.001. Data are expressed as means ± SD from three independent experiments performed in triplicate.

Changes of biological behavior in PHD2 CT26 and PHD2 MC38 cells

To assess the potential effects of overexpressing PHD2 on the biological behavior of colon cancer cells, the MTT, apoptosis, cell cycle, migration, and invasion assays were performed. As shown in Fig. 2A-B, compared with CT26 and MC38 cells, a significant proliferation inhibition of PHD2 CT26 and PHD2 MC38 cells was observed. In addition, the cell cycle analysis showed that the percentage of G1-phase cells was increased in PHD2 CT26 and PHD2 MC38 cells, along with decreasing expression of the cell cycle-associated proteins CyclinD1 and CDK4 (Fig. 2C-F). PHD2 overexpression was associated with decreased CyclinD1 and CDK4 in CT26 and MC38 cells (Fig. 2G-J). Interestingly, through Hochest333426 staining and apoptosis-associated protein detection, we revealed that there was almost no occurrence of apoptosis in PHD2 CT26 and PHD2 MC38 cells (Supp Fig. 1A-B). Moreover, the migration ability of CT26 and MC38 cells was suppressed by overexpressing PHD2 (Fig. 3A-C) and the expression levels of the EMT-associated protein Vimentin, and N-cadherin were down-regulated, while E-cadherin was up-regulated in PHD2 CT26 and PHD2 MC38 cells (Fig. 3D-G). Interestingly, in spite of the migration inhibition, overexpressing PHD2 has no influence on the change of invasive ability in CT26 and MC38 cells or on the expression of MMP2 and MMP9 (Supp Fig. 2).

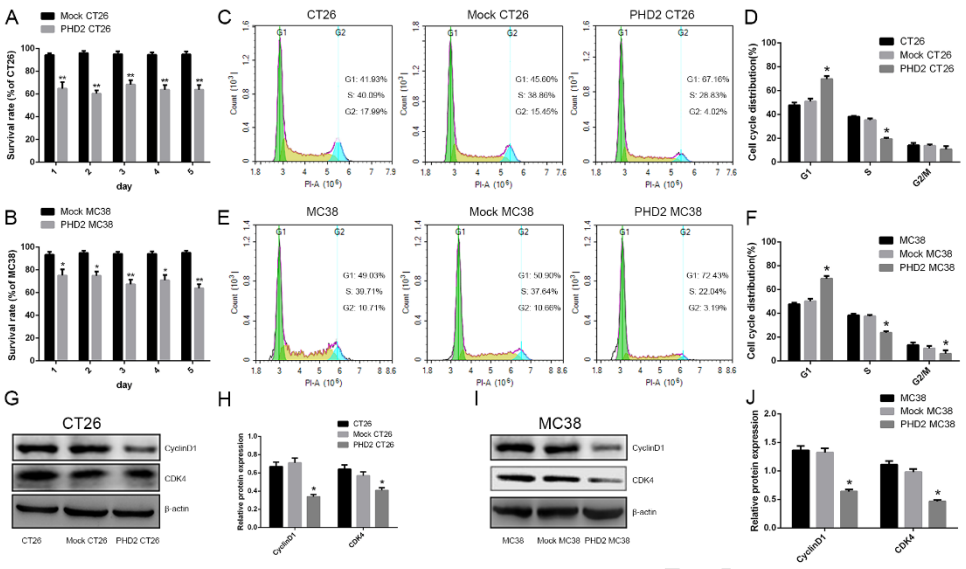


Figure 2. The effects of biological behavior in PHD2 CT26 and PHD2 MC38 cells. A. The MTT assay was performed to assess the effect of overexpressing PHD2 on CT26 cell proliferation. B. The MTT assay was performed to assess the effect of overexpressing PHD2 on MC38 cell proliferation. C. CT26 cell cycle arrest detection was carried out through flow cytometry. D. Quantification of C. E. MC38 cell cycle arrest detection was carried out through flow cytometry. F. Quantification of E. G. Western blot analysis of CyclinD1 and CDK4 in CT26 cells. H. Quantification of G. I. Western blot analysis of CyclinD1 and CDK4 in MC38 cells. J. Quantification of I. CT26 and MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. * P<0.05, ** P<0.01. Data are expressed as means ± SD from three independent experiments performed in triplicate.

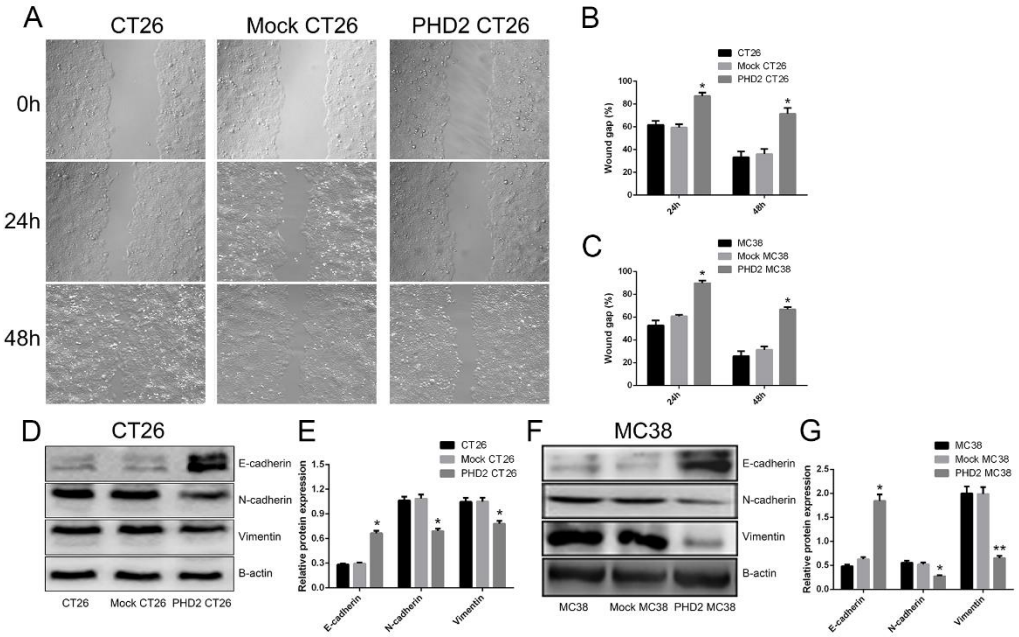
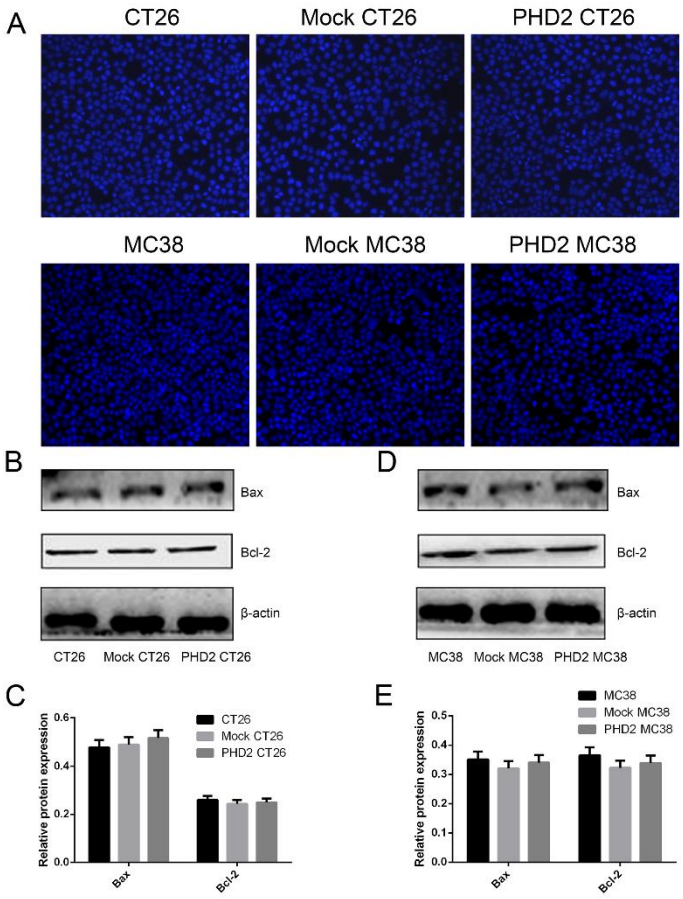
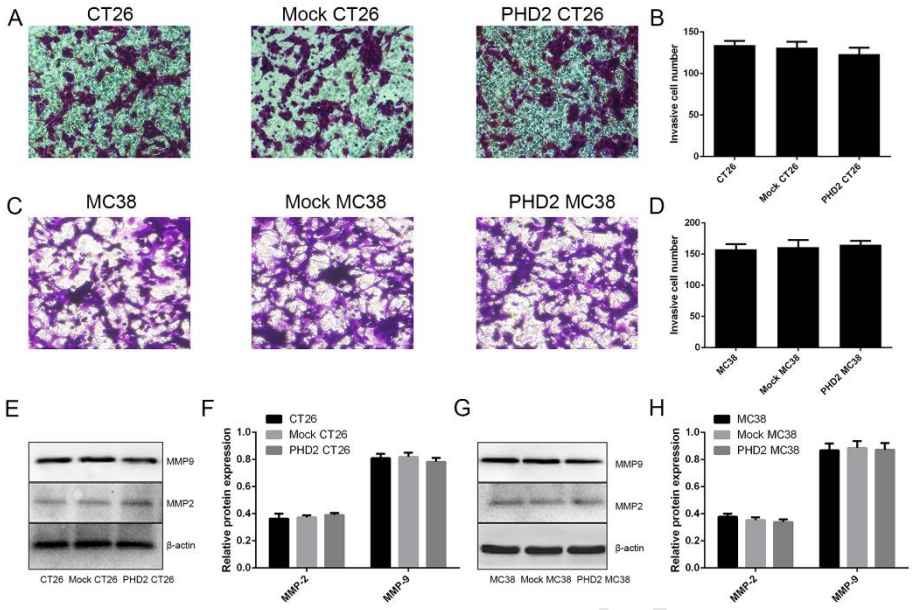


Figure 3. The wound-healing assay was conducted to investigate the effect of overexpressing PHD2 on cell migration. A. Representative wounds in time in CT26 cells. B. Quantification of A. C. Quantification of wound healing in MC38 cells. D. Western blot analysis of E-cadherin, N-cadherin, and Vimentin in CT26 cells. E. Quantification of D. F. Western blot analysis of E-cadherin, N-cadherin, and Vimentin in CT26 cells. G. Quantification of F. Original magnification: ×200 for all panels. CT26 and MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. * P<0.05, ** P<0.01. Data are expressed as means ± SD from three independent experiments performed in triplicate.



Supp Figure 1. Apoptosis induction of PHD2 CT26 and PHD2 MC38 cells. A. Hochest333426 staining was performed to assess cell apoptosis. B. Western blot analysis of Bcl-2 and Bax in CT26 cells. C. Quantification of B. D. Western blot analysis of Bcl-2 and Bax in MC38 cells. E. Quantification of D. Original magnification: ×200 for all panels. CT26 and MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. Data are expressed as means ± SD from three independent experiments performed in triplicate.



Supp Figure 2. The Transwell assay was conducted to evaluate the effect of overexpressing PHD2 on cell invasion. A. Migrated CT26 cells. B. Quantification of A. C. Migrated MC38 cells. D. Quantification of C. E. Western blot of MMP2 and MMP9 in CT26 cells. F. Quantification of E. F. Western blot of MMP2 and MMP9 in MC38 cells. G. Quantification of F. Original magnification: ×200 for all panels. CT26 and MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. Data are expressed as means ± SD from three independent experiments performed in triplicate.

Anti-cancer efficiency of PHD2 CT26 and PHD2 MC38 xenografts in mice

As we observed *in vitro*, overexpressing PHD2 inhibited the proliferation of CT26 and MC38 cells. We further explored the anti-cancer effects of PHD2 *in vivo*. As shown in Fig. 4A-C, we demonstrated that the tumor growth in PHD2 CT26 and PHD2 MC38 cell xenografts in mice was significantly suppressed, compared with CT26 and MC38 xenografts mice. Moreover, through immunofluorescence staining, we observed that the expression of Ki67 in PHD2 CT26 and PHD2 MC38 xenografts was decreased (Fig. 4D).

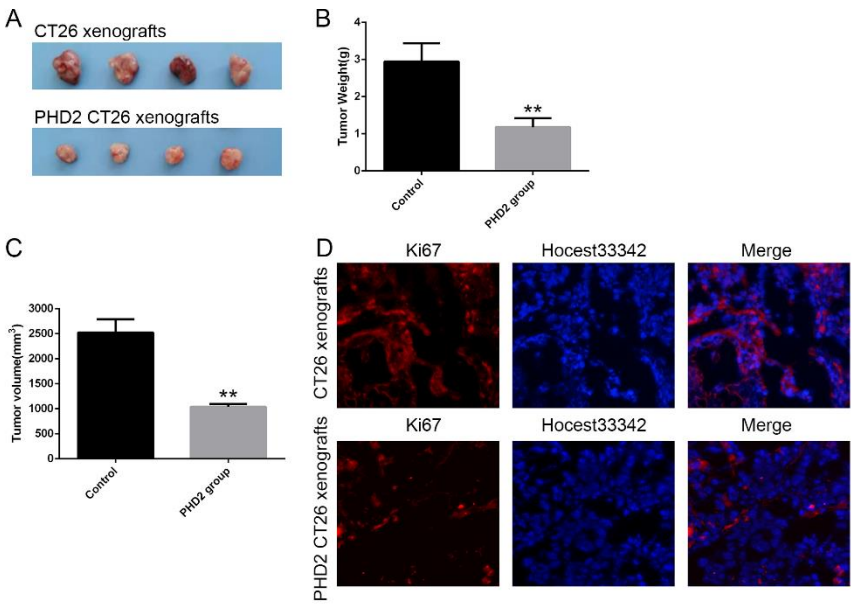


Figure 4. Tumor growth of PHD2 CT26 and PHD2 MC38 xenografts in mice. A. Tumor tissues derived from PHD2 CT26 and PHD2 MC38 xenografts or CT26 and MC38 xenografts. B-C. Tumor weights and tumor volumes were examined until 5 weeks after inoculation. D. Immunofluorescence staining of Ki67 in tumor tissues. Original magnification: ×200 for all panels. Control *vs.* PHD2 group. ** P<0.01. Data are expressed as means ± SD (n=4 for each group).

Pro-inflammatory cytokines levels in PHD2 CT26 and PHD2 MC38 cancer xenograft mice

In order to explore the effects of PHD2 on inflammatory responses in colon cancer xenografts, we analyzed a series of pro-inflammatory cytokines. As shown in Fig. 5, we verified that the secretory levels of G-CSF, TNF-α, IL-6, IL-4, IL-1β, and IL-8 were attenuated in PHD2 CT26 and PHD2 MC38 cell xenografts compared with CT26 and MC38 xenografts, especially regarding TNF-α, IL-1β, and G-CSF levels.

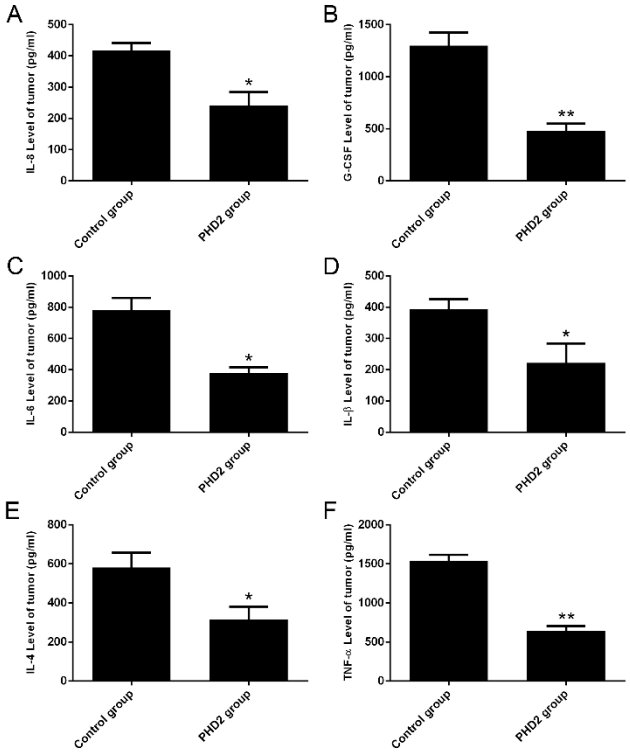


Figure 5. The changes in pro-inflammatory cytokines levels in PHD2 CT26 and PHD2 MC38 xenografts. Elisa assay was conducted to detect the secreted levels of G-CSF, IL-6, IL-8, IL-1β, TNF-α, and IL-4. Control group *vs.* PHD2 group. * P<0.05, ** P<0.01. Data are expressed as means ± SD (n=4 for each group).

MDSCs and M2-like TAMs proportions in PHD2 CT26 and PHD2 MC38 xenografts in mice

In addition to analyzing the pro-inflammatory cytokines, we investigated the primary inflammatory cells, including MDSCs and M2-like TAMs. Through flow cytometry analysis, we detected these cells in xenografts derived from the aforementioned groups. The results showed that the proportions of MDSCs and M2-like TAMs were obviously decreased in PHD2 CT26 and PHD2 MC38 cell xenografts compared with CT26 and MC38 xenografts (Fig. 6).

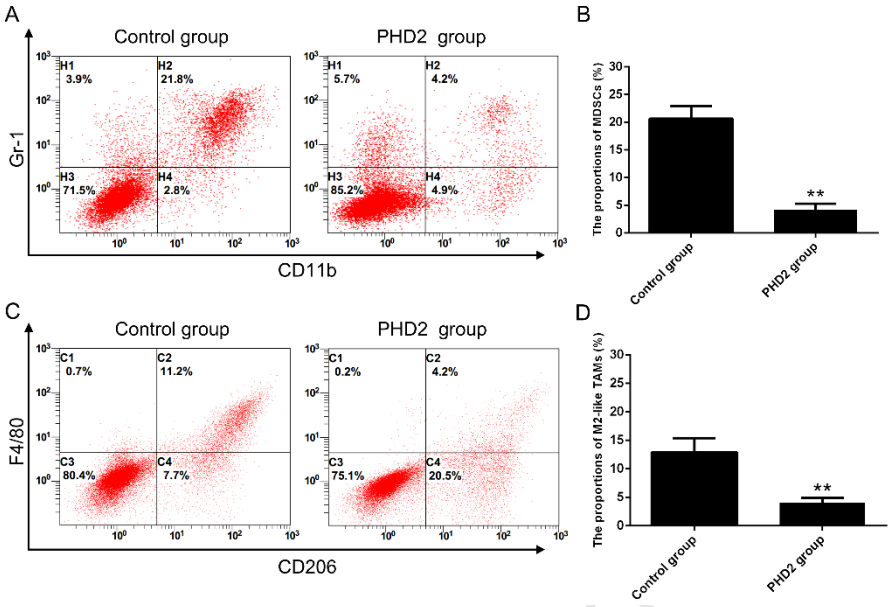


Figure 6. The changes of MDSCs and M2-like TAMs proportions in PHD2 CT26 and PHD2 MC38 xenografts. The MDSCs (A-B) and M2-like TAMs (C-D) proportions in tumor tissues were assessed using flow cytometry. Control group *vs.* PHD2 group. ** P<0.01. Data are expressed as means ± SD (n=4 for each group).

Overexpressing PHD2 suppressed NF-κB activity in colon cancer cells

To elucidate the underlying molecular mechanisms, western blot analysis was performed to investigate the NF-κB signaling pathway. We demonstrated that in PHD2 CT26 and PHD2 MC38 cells, the expression of cytoplasmic NF-κB p65 was increased, while phosphorylated NF-κB p65 was reduced, compared with CT26 and MC38 cells. Meanwhile, overexpressing PHD2 decreased phosphorylated IκB-α while increased IκB-α level in colon cancer cells (Fig. 7A-D). In addition, the levels of nuclear phosphorylated NF-κB p65 and NF-κB p65 were both decreased in PHD2 CT26 and PHD2 MC38 cells (Fig. 7E-H). Notably, in xenografts, we observed that overexpressing PHD2 significantly reduced the expression levels of phosphorylated NF-κB p65 and phosphorylated IκB-α (Fig. 7I-J). Furthermore, we analyzed pro-inflammatory cytokines in PHD2 CT26 and PHD2 MC38 cells and found that the secretory levels of TNF-α and G-CSF were decreased compared with CT26 and MC38 cells (Fig. 7K-L).

PDTC, an inhibitor of NF-κB, was used to clarify the NF-κB activity regulated by

PHD2 and its involvement in anti-cancer and anti-inflammatory effects. As expected, the expression levels of CyclinD1 were down-regulated, while E-cadherin was up-regulated in colon cancer cells after being treated with PDTC. The expression of the pro-inflammatory cytokine TNF-α was also decreased (Fig. 7M-Q). Interestingly, we found that TNF-α synergy with G-CSF promoted the migration of MDSCs (Fig. 7R-S).

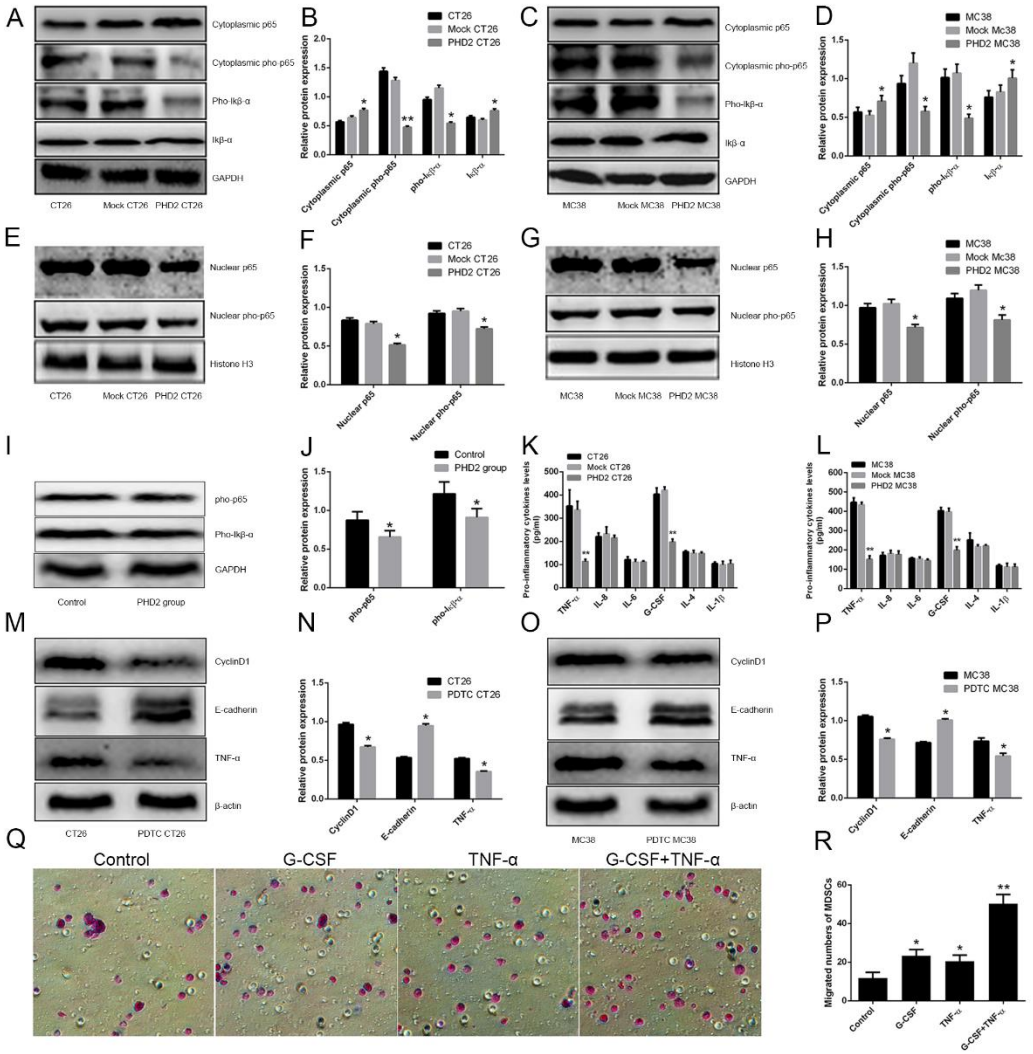


Figure 7. The effects of overexpressing PHD2 on NF-κB activity *in vitro* or *in vivo*. A. Western blot analysis of cytoplasmic phosphorylated p65, p65, phosphorylated IκB-α, and IκB-α in CT26 cells. B. Quantification of A. C. Western blot analysis of cytoplasmic phosphorylated p65, p65, phosphorylated IκB-α, and IκB-α in MC38 cells. D. Quantification of C. E. Western blot analysis of nuclear phosphorylated p65 and p65 in CT26 cells. F. Quantification of E. G. Western blot analysis of nuclear phosphorylated p65 and p65 in MC38 cells. H. Quantification of G. CT26 and

MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. Data are expressed as means ± SD from three independent experiments performed in triplicate. I. Western blot analysis of phosphorylated p65 and phosphorylated IκB-α in tumor tissues. J. Quantification of I. Control *vs.* PHD2 group. Data are expressed as means ± SD (n=4 for each group). K. ELISA assays were conducted to detect the secreted levels of G-CSF, IL-6, IL-8, IL-1β, TNF-α, and IL-4 in CT26 cells. L. ELISA assays were conducted to detect the secreted levels of G-CSF, IL-6, IL-8, IL-1β, TNF-α, and IL-4 in MC38 cells. CT26 and MC38 cells *vs.* Mock CT26 and Mock MC38 cells or PHD2 CT26 and PHD2 MC38 cells. M. Western blot analysis of CyclinD1, E-cadherin, and TNF-α in CT26 cells treated with or without PDTC (100 μmol/L). O. Quantification of M. P. Western blot analysis of CyclinD1, E-cadherin, and TNF-α in MC38 cells treated with or without PDTC (100 μmol/L). Q. Quantification of P. Colon cancer cells *vs.* PDTC-colon cancer cells. R. The Transwell assay was conducted to evaluate MDSCs migration in the presence of TNF-α (100 ng/mL) or/and G-CSF (10 nM). S. Quantification of R. Original magnification: ×200 for all panels. Control *vs.* G-CSF or TNF-α or G-SCF combined with TNF-α. * $P<0.05$, ** $P<0.01$. Data are expressed as means ± SD from three independent experiments performed in triplicate.

Discussion

Recent reports have already verified the existence of "crosstalk" between the tumor microenvironment and tumor progression (Yuan et al. , 2016). Improving tumor microenvironment, including attenuating exacerbated inflammatory or/and immunosuppressive conditions, dramatically suppresses tumor growth in many types of tumor-bearing mice models (Maletzki et al. , 2019, Ren et al. , 2019). Therefore, previous anti-cancer strategy only targeting cancer cells is insufficient, while aiming to the cancer cell and tumor microenvironment seems to be necessary and imperative to explore.

PHD2 has previously been reported to regulate HIF-1α levels in normoxia via hydroxylation for proteasomal degradation. Recently, some researchers indicated that the loss of PHD2 in *Streptococcus pneumonia* infection resulted in an exaggerated inflammatory response (Sadiku, Willson, 2017). Another study revealed that PHD2

knockdown suppressed LPS-induced inflammatory response in periodontitis (Fan, Mao, 2019). In colon cancer, PHD2 is considered to be a tumor suppressor (Rawluszko, Bujnicka, 2013), but its association with tumor progression involved with intratumoral inflammatory regulation and the underlying molecular mechanism have not been totally uncovered. Therefore, in this study, we constructed PHD2-overexpressing colon cancer cells and established PHD2-overexpressing colon cancer xenografts in mice to elucidate the effects of PHD2.

Firstly, we constructed PHD2 CT26 and PHD2 MC38 cells and observed a significant proliferation inhibition in these cells, which is similar to a previous report in hepatocellular carcinoma (Tao, Lin, 2016). Studies suggested that cell cycle arrest and apoptosis induction were closely associated with the inhibition of proliferation in cancer cells (Kassab and Hassan, 2018). On the other hand, Cell cycle and apoptosis are linked to some extent, but they involve different pathways and mechanisms (Marretta and Ales, 2010, Pucci et al. , 2000, Santos et al. , 2013). In this study, we demonstrated that overexpressing PHD2 promoted cycle arrest of colon cancer cells. CyclinD1 is an important regulator of the cell cycle. By activating its partner kinase, CDK4, elevated CyclinD1 drives the G1 to S phase transition of cancer cells (Davis et al. , 2016). Our results showed that overexpressing PHD2 down-regulated the expression levels of CyclinD1 and CDK4 and arrested the cell cycle progression. A previous study in mice showed that the loss of PHD2 leads to the induction of stem cells (Singh et al. , 2013). In cancer cells of endothelial origin, suppression of PHD2 leads to decreased proliferation (Chan et al. , 2009). In liver cancer, inhibition of PHD2 arrests the cell cycle and decreases the expression of cyclin D1, supporting the present study (Tao, Lin, 2016). Interestingly, there was almost no occurrence of apoptosis in PHD2 CT26 and PHD2 MC38 cells. Accordingly, there were no differences in Bax (an apoptosis activator) and Bcl2 (an apoptosis inhibitor) levels among the groups of cells. A study in breast cancer cells showed that in the context of glucose starvation, PHD2 induces apoptosis, and its silencing rescue the cells from apoptosis (Di Conza et al. , 2017b). On the other hand, a study in cisplatin-resistant ovarian cancer suggests that PHD2 is phosphorylated by ERK, a major factor

involved in apoptosis and that PHD2 might be downstream of major apoptosis factors instead of upstream (Mebratu and Tesfaigzi, 2009). Those results indicate that the induction of cell cycle arrest might be the major form of PHD2-induced anti-proliferation effect. Therefore, the effect of PHD2 on the proliferation of cancer cells seems only to involve cell cycle arrest and not apoptosis. In fact, the role of PHD2 in apoptosis is probably dependent upon the hypoxic state of the cells (Bordoli et al. , 2011), but this was not investigated in the present study. Nevertheless, PHD2 is an effector in the presence of inflammation (Fan, Mao, 2019, Sadiku, Willson, 2017), and the cells were cultured in the absence of inflammatory stimuli. Therefore, additional studies are necessary to determine the exact role of PHD2 in cancer cell proliferation, especially because PHD2 can inhibit or accelerate tumor progression, depending upon the cancer type (Ortmann et al. , 2014). Of note, high levels of PHD2 is associated with good prognosis in patients with colon cancer, hepatocellular carcinoma, and melanoma (Di Conza, Trusso Cafarello, 2017a, Rawluszko, Bujnicka, 2013, Tao, Lin, 2016), but PHD2 is associated with cisplatin resistance in ovarian cancer (Mebratu and Tesfaigzi, 2009), suggesting different roles in different cancers or upon various stimuli (e.g., cytotoxic agents).

Metastasis is the main cause of the mortality rates of colon cancer patients. Two cellular processes are involved: migration (cell displacement in the absence of obstacles) and invasion (cell displacement in the presence of obstacles). Cell invasion requires enzymes (such as matrix metalloproteinases (MMP)) that digest the extracellular matrix to allow the cells to pass through (Webb et al. , 2017). MMPs also play roles in angiogenesis required for tumor growth (Webb, Gao, 2017). EMT is a concept in which epithelial cells undergo a phenotypic switch to acquire the mesenchymal phenotype, which is involved closely with invasion (Sun et al. , 2019). In this process, epithelial markers such as E-cadherin are reduced. Meanwhile, mesenchymal markers (such as N-cadherin and Vimentin) are increased, and cells are loosely arranged, with strong mobility. The EMT is a critical event in cancer cell dissemination and hematogenous spreading (Banyard and Bielenberg, 2015, Roche, 2018). In the present study, we showed that PHD2 overexpression decreased the EMT

of the PHD2 CT26 and PHD2 MC38 cells, as shown by downregulated N-cadherin and Vimentin expression and upregulated E-cadherin expression. This inhibitory effect of PHD2 on EMT has been reported in many cell types and involves the TGF-β and SMAD2/3 pathways (Han et al. , 2013, Serrano-Gomez et al. , 2016). Those changes resulted in a decreased migration ability of the cells. On the other hand, the invasion ability of the cells was not changed by PHD2 overexpression, as shown by the Transwell assay and by similar levels of MMP2 and MMP9 among the groups. This result is difficult to explain in the context where both migration and invasion are features of EMT, which was inhibited here by PHD2 overexpression. The exact reasons cannot be drawn from the present study because it was not designed to explore the variety of pathways involved. Additional studies will have to examine this point.

Next, we established PHD2-overexpressing colon cancer xenografts in mice models to explore the anti-cancer efficiency *in vivo*. We verified that tumor growth was significantly inhibited in PHD2-overexpressing colon cancer-bearing mice. Moreover, we detected the expression level of Ki67 in xenografts, which is an important proliferative index and commonly used in many types of cancer, including colon cancer (Gurzu et al. , 2007). As expected, the Ki67 levels were significantly decreased in PHD2-overexpressing colon cancer-bearing mice, consistent with the changes in proliferation inhibition in the PHD2-overexpressing colon cancer cells, as verified *in vitro*.

We investigated the possible changes in inflammatory responses in colon cancer-bearing mice. The pro-inflammatory cytokines TNF-α, G-CSF, IL-8, IL-4, IL-1β, and IL-6, play major roles in the inflammatory response and are closely involved with the occurrence and development of colon cancer (Ong et al. , 2010, Scheller et al. , 2014). In the present study, in PHD2-overexpressing colon cancer xenografts, the levels of the aforementioned cytokines were decreased compared with colon cancer xenografts in mice.

MDSCs, a class of immature myeloid cells, are described as a subset of tumor-associated inflammatory cells and are recruited to the tumor microenvironment

by pro-inflammatory cytokines such as TNF-α, G-CSF, GM-CSF, and IL-8 secreted by cancer cells or stromal cells (Chiu et al. , 2016). Accumulation of MDSCs promotes the progression of inflammatory responses through producing IL-6 and IL-1β (Bruchard et al. , 2013), which promote the transformation of normal cells to malignant cells. TAMs are another well-described population of myeloid cells originating from mononuclear phagocytic lineage. M2-like TAMs infiltration in the tumor microenvironment and promotes substantial secretion of pro-inflammatory cytokines such as TNF-α and IL-12, which are closely related to poor prognosis of cancer (Tariq, Zhang, 2017). Based on these, we analyzed the proportions of MDSCs and M2-like TAMs in xenografts. As expected, in PHD2-overexpressing colon cancer xenografts, the proportions of these cells were obviously lower compared with colon cancer xenografts.

NF-κB is a well-known factor associated with cell cycle regulation and inflammation, promoting cell growth in the presence of inflammation (Lemieszek et al. , 2019). In the present study, overexpression of PHD2 suppressed the constitutive NF-κB activation by blocking the phosphorylation of IκB-α. In addition, the levels of TNF-α and G-CSF were down-regulated in PHD2-overexpressing colon cancer cells, while almost no effects were observed on the levels of IL-8, IL-4, IL-1β, and IL-6, indicating that these pro-inflammatory cytokines might be regulated by other cells in the tumor microenvironment, such as by tumor-associated fibroblasts or natural killer cells (Littwitz-Salomon et al. , 2018, Liubomirski et al. , 2019). We used an NF-κB inhibitor to investigate the downstream genes of NF-κB involved in the cell cycle, EMT, and inflammation, including CyclinD1, E-cadherin, and TNF-α. The results showed that these proteins were changed in colon cancer cells after being treated with the NF-κB inhibitor. TNF-α or G-CSF can increase the migration of MDSCs in the tumor microenvironment and promote tumor growth (Li et al. , 2016, Zhang et al. , 2015). Indeed, through transwell assay, we observed that TNF-α synergy with G-CSF could significantly accelerate the migration of MDSCs. All these results demonstrated that the anti-inflammatory effect of PHD2 was exerted through decreasing NF-κB activity, which further attenuated the secretion of TNF-α and G-CSF from colon

cancer cells and then suppressed the recruitment of inflammatory cells in the tumor microenvironment. This might be closely involved with the inhibition of tumor growth. Fig. 8 provides a schematic representation of the results of this study.

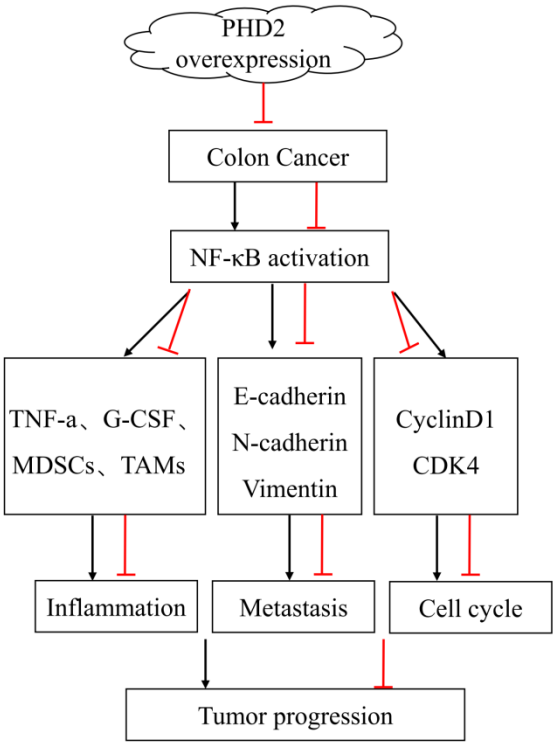


Figure 8. Schematic representation for the outcome of this study.

Conclusions

The present study suggests that PHD2 exerts anti-cancer and anti-inflammatory effects in colon cancer cells. Overexpression of PHD2 arrested cell cycle, inhibited EMT, inhibited cell migration, and alleviated the inflammatory tumor microenvironment, but without effect on apoptosis and cell invasion. The exact mechanisms of PHD2 involvement still remain to be defined.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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